

# Juvenile hormone III titers and regulation of soldier caste in *Coptotermes formosanus* (Isoptera: Rhinotermitidae)

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## Abstract

Juvenile hormone (JH) is an important growth hormone in insects that has also been implicated in caste determination in termites. Gas chromatography–mass spectrometry was used to establish that the JH in the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is JH III. JH III titers were measured in workers, pre-soldiers, and soldiers from samples collected from the field. The average titers of JH III in workers and soldiers were about 13 and 25 pg mg<sup>−1</sup>, respectively. However, pre-soldiers contained a significantly higher amount, 596 pg mg<sup>−1</sup>. As expected, treatment of workers with a JH-analogue, methoprene, triggered rapid formation of pre-soldiers. However, these pre-soldiers had a very low JH III titer (62 pg mg<sup>−1</sup>). It appears that the application of JHA, while inducing pre-soldier formation, does not increase the endogenous JH III titer. The titer, however, increased as the pre-soldiers aged and before transforming into soldiers.

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## 1. Introduction

The developmental pathway of the various castes in termites, from larva to “worker” or pre-soldier/soldier or nymph/imago, is strongly influenced by the changes in juvenile hormone (JH) titer. It has been shown that the concentrations of JH or JH-analogue (JHA) are known to play a key role in caste differentiation for many termite species (Hrdý and Křeček, 1972; Lüscher, 1972; Wanyonyi, 1974; Haverty and Howard, 1979; Howard and Haverty, 1979; Hrdý et al., 1979; Okot-Kotber, 1980a, 1980b; Nijhout and Wheeler, 1982; Jones, 1984; Su et al., 1985; Varma, 1985; Lelis and Everaerts, 1993). Workers with a low JH titer remain as workers, even after molt, whereas workers with high JH titer or after application of JHA undergo differentiation into pre-soldiers/soldiers (Wanyonyi and Lüscher, 1973; Wanyonyi, 1974). In *Coptotermes*

*formosanus* Shiraki, JH III or JHA appeared to induce soldier differentiation (Park and Raina, 2003).

In *C. formosanus*, the workers that have 11 antennal segments or more have a potential to differentiate into pre-soldiers (Park, unpublished). It took 10–12 days at 28 ± 1 °C for pre-soldiers to molt into soldiers (Wang et al., 2002; Park and Raina, 2003). Further, the frequency of transformation of workers to soldiers in this species increased in the absence of soldiers and then declined when the colony attained a certain population ratio of soldiers (Haverty, 1979; Park and Raina, 2003). It has been suggested that different termite species have different proportions of soldiers (Haverty, 1977; Thorne, 1985; Waller and La Fage, 1987). Previous researchers postulated that transformation of workers to soldiers might be controlled by soldiers releasing compounds, including anti-JH substances or pheromones (Noirot, 1969; Springhetti, 1970; Lüscher, 1972; Lefeuvre and Bordereau, 1984).

All previous investigations, however, have been confined only to treatment with JHAs in relation to soldier formation in termites. There is no information available

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on how various termite castes regulate their hormone titers in the field. One of the critical questions is how workers are differently influenced in the absence or presence of soldiers in the population, as well as in relation to the different soldier–population ratio of the colony. We, thus, investigated the profiles of JH III titers in workers, pre-soldiers, and soldiers collected from field colonies to better explain its physiological or hormonal effects on *C. formosanus*. We also determined periodic hormonal changes in termite workers with or without the presence of soldiers. Finally, the hormonal effect of exogenous JHA on workers was investigated with the treatment of a JHA in order to trigger the transformation of worker to soldier.

## 2. Materials and methods

Formosan subterranean termites were collected from three different locations in Orleans Parish, LA: two colonies from City Park and one colony from the University of New Orleans. Termites were sorted into groups of workers, pre-soldiers and soldiers immediately after collection from the field. For JH titer analysis, the samples were prepared in three replicates, each representing 900 workers and 50 soldiers. The number of pre-soldiers used for sample extraction was not consistent for the three collected colonies due to the scarcity of pre-soldiers in collections. All termite samples were weighed before processing. An internal standard, containing 2 ng of JH III ethyl ester in acetonitrile, was added to all samples. Samples were analyzed for JH titers by gas chromatography–mass spectrometry (GC–MS) according to Bergot et al. (1981) with modifications (Shu et al., 1997). Samples were homogenized in a mixture of hexane, acetonitrile, and 2% NaCl (2:1:1 in volume ratio), and centrifuged at 400g for 10 min (IEC, Needham Heights, MA). The supernatant of each centrifuged sample was transferred into a clean vial. The centrifuge-separation process was repeated twice for each sample. The supernatants were combined and concentrated in a SpeedVac (SVC 100H; Savant Instruments Inc.). The residue was resuspended in 200  $\mu$ l hexane and stored in  $-80^{\circ}\text{C}$  until use. Each sample and a rinse of 200  $\mu$ l hexane were loaded on activated  $\text{Al}_2\text{O}_3$  column (treated with 6% water) and eluted with hexane, 10% diethyl ether in hexane, and 30% E/H to recover JH III. The JH-containing 30% E/H fraction was dried in the SpeedVac. The residue was resuspended with 75- $\mu$ l  $d_4$ -methanol, then 75  $\mu$ l 5% trifluoroacetic acid in  $d_4$ -methanol to obtain  $d_3$ -methoxyhydrin derivatives of JHs. The solution was derivatized in an oven at  $60^{\circ}\text{C}$  for 20 min. After the sample was dried in the SpeedVac, the residue was dissolved in 200  $\mu$ l hexane. The solutions were eluted through the activated  $\text{Al}_2\text{O}_3$  column with hexane, 30% E/H, and

50% ethyl acetate in hexane to recover the  $d_3$ -methoxyhydrin derivatives. The 50% ethyl acetate in hexane fractions was dried in the SpeedVac. Residues were resuspended with hexane and again dried in the SpeedVac. The residue was dissolved in 100  $\mu$ l hexane and taken up in 2  $\mu$ l hexane and subjected to GC–MS analysis.

The samples were detected in a selected ion mode. The  $d_3$ -methoxyhydrin derivative of JH III was monitored for fragments at  $m/z$  76 and 225;  $d_3$ -methoxyhydrin derivatives of JH II and JH I were monitored for fragments at  $m/z$  90 and 225 and  $m/z$  90 and 239, respectively. The internal standard, JH III-ethyl, was monitored for  $m/z$  76 and 239. Total abundance of specific JHs was quantified against that for the internal standard (Shu et al., 1997). JH quantity was normalized and expressed as  $\text{pg mg}^{-1}$  of wet body weight.

An experiment was conducted to determine the effect of the presence or absence of soldiers on workers. One group was composed of 550 workers without soldiers (0%) while the other 500 workers and 233 soldiers (32%). The latter percentage of soldiers was most effective for feedback inhibition of additional soldier formation in *C. formosanus* (Park and Raina, 2003). Each group was placed in a Petri dish (100  $\times$  15 mm, Falcon) with four moistened pine veneers (4  $\times$  4 cm). The numbers of pre-soldiers and soldiers formed were recorded after 40 and 80 days.

Based upon the results of the above experiment and results obtained previously (Park and Raina, 2003), hormonal changes in workers were predicted to appear about 20 days after the workers are separated from the soldiers. In order to determine the changes in the JH titers of workers while producing pre-soldiers, the existing pre-soldiers and soldiers were removed from workers of three different colonies. Six thousand workers from each colony were placed in plastic containers (17  $\times$  12  $\times$  6.5 cm) with four moistened southern yellow pine blocks (9.7  $\times$  3.8  $\times$  0.5 cm). At 16-day intervals for a total of 48 days, numbers of workers, pre-soldiers, and soldiers in each group were counted and 900 workers were removed from each container, to determine the JH titer.

To examine the effect of an exogenous JHA, methoprene (Chem Service, West Chester, PA), on JH III titer in pre-soldiers, 20 workers from each of the three colonies were placed in a 50  $\times$  9 mm Petri dish containing a filter paper (P5, Fisher Sci., Suwanee, GA) impregnated with 4  $\mu$ g of the JHA in acetone (B&J). Acetone treated filter paper served as the control. Three different types of pre-soldiers: <3 days and 5–7 days old from treated groups, and <3 days old from control group, were removed and stored at  $-80^{\circ}\text{C}$  until at least 10 pre-soldiers were accumulated for each sample. JH titers were determined as described above.

Because the JH titer in pre-soldiers produced from workers treated with methoprene was significantly lower than in normally produced pre-soldiers, we investigated whether JH titer of the former would subsequently rise while they develop from pre-soldiers to soldiers. To do that, methoprene treated and untreated workers were set up as above. The age of pre-soldiers used for JH extraction ranged from 5 to 7 days old. JH titers were determined as previously described.

### 3. Results

We report for the first time that JH III is the only JH homologue found in workers, pre-soldiers, and soldiers of *C. formosanus*. JH III titers in workers, pre-soldiers and soldiers are presented in Fig. 1. The JH III titers of the workers and soldiers were  $12.9 \pm 3.4$  and  $24.8 \pm 4.6$  pg mg<sup>-1</sup>, respectively. The titer of the pre-soldiers was  $596.4 \pm 95.6$  pg mg<sup>-1</sup>, about 45 times higher than that of the workers. After transformation from pre-soldiers to soldiers, the JH titer declined by about 20-fold relative to that of pre-soldiers.

No pre-soldiers were produced in the group of workers with a soldier ratio of 32% while the group without soldiers produced 15% pre-soldiers in the first 40 days (Table 1). The results clearly indicated that the presence of a high proportion of soldiers (32%) in the colony negatively affected additional pre-soldier production. The pre-soldiers molted into soldiers in 10–12 days under laboratory conditions.

In another experiment involving groups of 6000 workers and no soldiers, the percentages of pre-soldiers

and of soldiers were  $3.44 \pm 1.68$  and  $0.06 \pm 0.04$ , respectively, after 16 days (Fig. 2). After 32 days the cumulative percentage of pre-soldiers increased to  $7.86 \pm 3.24$  and the percentage of soldiers also increased to  $7.76 \pm 2.72$ . By 48 days, the formation of additional pre-soldiers decreased significantly ( $1.30 \pm 0.87\%$ ), while the existing pre-soldiers molted to soldiers increasing their percentage to  $23.40 \pm 6.43$ . The JH III titer in workers at each observation point showed an initial increase from  $9.73 \pm 6.41$  to  $42.97 \pm 25.91$  pg mg<sup>-1</sup> and then decreased to  $28.04 \pm 7.50$  pg mg<sup>-1</sup> at 32 days and subsequently to  $18.87 \pm 6.36$  pg mg<sup>-1</sup> at 48 days. The results indicated that the JH III titer in workers was influenced by the proportion of soldiers (and perhaps pre-soldiers also) in the population.

To determine the JH titer in pre-soldiers formed from workers with and without treatment with a JHA, one group of workers was treated with methoprene. There was a 10-fold lower JH titer in <3 days old pre-soldiers ( $61.54 \pm 35$  pg mg<sup>-1</sup>) produced from workers that were treated with the JHA compared to the control group (Fig. 3). However, when pre-soldiers formed in response to methoprene treatment were allowed to stay until they became 5–7 days old pre-soldiers, the JH titer increased to about  $407.26 \pm 212.83$  pg mg<sup>-1</sup>.

### 4. Discussion

A mature Formosan subterranean termite colony consists of different castes: larvae, workers, pre-soldiers, soldiers, brachypterous line (nymphs), alates, and

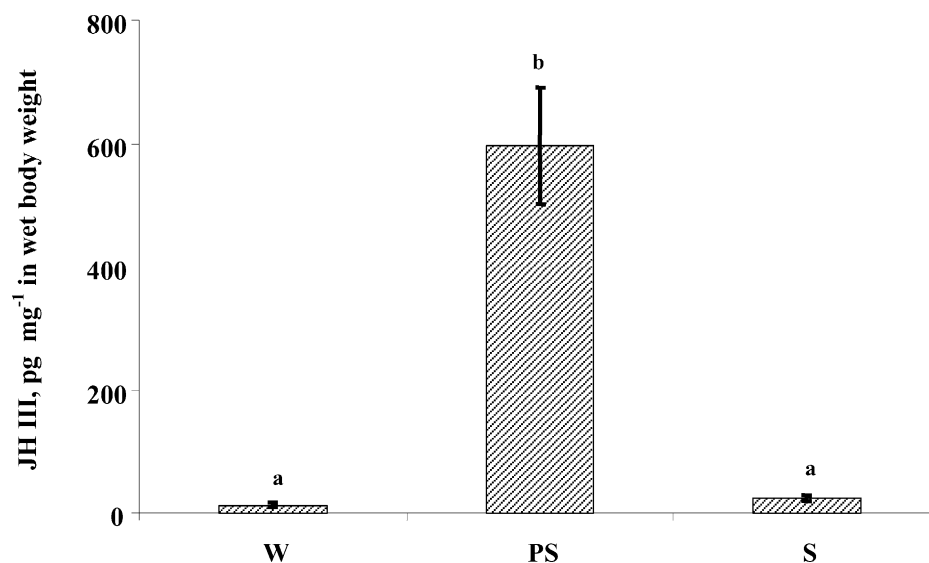


Fig. 1. Juvenile hormone III titers of workers (W), pre-soldiers (PS), and soldiers (S) of *C. formosanus*, extracted immediately after collection from the field. Means are of three replicates from each of the three colonies. Bars represent standard errors (SE). Bars with the same letter are not significantly different at  $P < 0.05$  by one-way ANOVA followed by Tukey's multiple comparison test.

Table 1

Formation of pre-soldiers and soldiers from workers of *C. formosanus* with and without the presence of soldiers after 40 and 80 days

Days	Percent pre-soldiers and soldiers formed			
	In absence of soldiers		In presence of soldiers	
	Pre-soldiers	Soldiers	Pre-soldiers	Soldiers
40	10.5 ± 5.4	5.6 ± 2.2	0.0 ± 0.0	34.9 ± 0.2
80	0.0 ± 0.0	25.0 ± 1.8	0.0 ± 0.0	32.6 ± 1.9

primary or secondary (replacement) reproductives. As the terminology indicates, they show a diversity of morphological characteristics and function. The colony appears to maintain balanced caste ratios through regulation of their hormonal titers (unpublished data). Hormonal regulation of caste differentiation in termites has been studied mainly for the formation of soldiers from workers. We have shown for the first time that the JH in *C. formosanus* is JH III. The determination of JH III came from monitoring unique MS fragment ions ( $m/z$  76 and 225) at specific retention time. To ensure absence of JH I and JH II in the whole body extracts of worker, pre-soldier, and soldier, full-scan GC–MS spectra of each caste were monitored. There were no specific MS fragment ions for JH I ( $m/z$  90 and 239) and JH II ( $m/z$  90 and 225) in *C. formosanus* (data not shown).

JH titers of workers as these change to pre-soldiers and then to soldiers have not been determined previously. JH III titer was low in workers freshly collected from the field. Because it was very difficult to predict which workers were going to molt into pre-soldiers, it was not possible to determine the JH titer of workers destined to become pre-soldiers in a colony. Based on the established role of hormones in insect growth and development, we speculated that the JH

titer of such workers would be high. As shown in Fig. 2, the group of workers prospective to become pre-soldiers/soldiers showed higher JH titers in *C. formosanus*. However, pre-soldiers, being a transition stage, had a JH titer that was almost 45 times higher than that in workers. Park and Raina (2003) showed that the corpora allata, the site of JH production, of pre-soldiers were enlarged in size when compared to those of workers and soldiers in *C. formosanus*. The JH titer in soldiers was also low, although interestingly not as low as that of workers.

Henderson and Rao (1993) suggested that the workers in *C. formosanus* have a capability to molt into soldiers when more soldiers are needed to defend the colony during a specific period, including the swarming season. Other factors that may induce formation of additional soldiers are low soldier numbers, physical disturbance to a colony in its original location and nutrition.

In *C. formosanus*, the formation of additional soldiers is controlled by existing soldiers rather than primary or secondary reproductives (Park and Raina, 2003), while in *Kaloterme flavicollis*, it can be regulated by the number of workers or pseudergates (Springhetti, 1973). Removing soldiers resulted in hormonal changes in workers. The hormonal regulation of

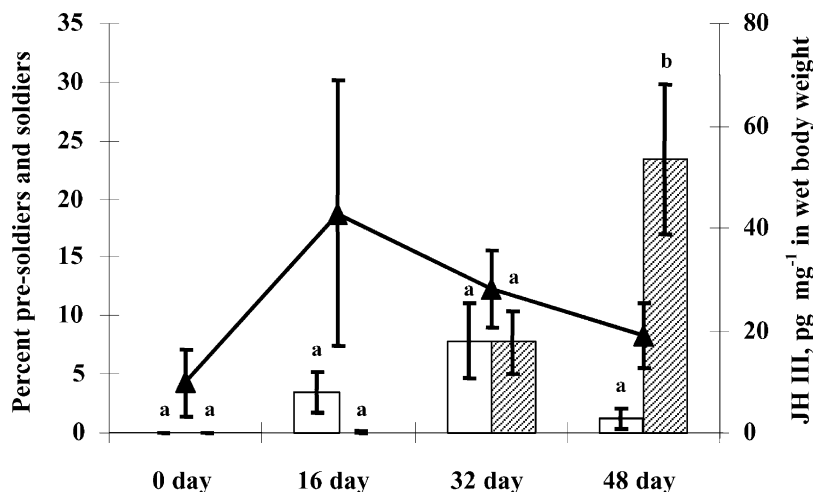


Fig. 2. Percent of pre-soldiers (□) and soldiers (▨) of *C. formosanus* formed every 16 days in 6000 workers after all soldiers had been removed (bar), and juvenile hormone titers of workers at each observation day (—▲—). Means ± SE are of three replicates each representing a different colony. Bars with the same letter are not significantly different at  $P < 0.05$  by one-way ANOVA followed by Tukey's multiple comparison test.

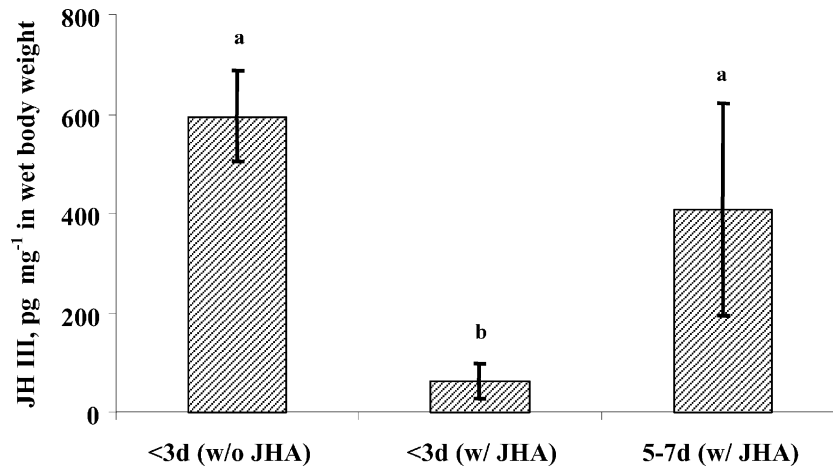


Fig. 3. Changes in JH III titer in pre-soldiers of *C. formosanus* formed in response to treatment of workers with 4  $\mu$ g methoprene on filter paper. Means  $\pm$  SE represent three replicates each of three colonies. Bars with the same letter are not significantly different at  $P < 0.05$ , one-way ANOVA followed by Tukey's multiple comparison test.

soldier production was maintained until the colony could establish an optimal soldier percentage. In our experiment with high soldier percentage (32%) present in the colony, no additional pre-soldiers were formed after 40 and 80 days, suggesting that there was a strong inhibition of further differentiation into pre-soldiers. However, in the group that had no soldiers, workers could produce pre-soldiers and soldiers that reached  $10.5\% \pm 5.4\%$  and  $5.6\% \pm 2.2\%$ , respectively, in 40 days. After 80 days, the percentage of soldiers reached about 25%. However, no pre-soldier was present at that time indicating that the presence of soldiers suppressed the intrinsic hormonal changes in workers. In our previous work, we had shown that when workers were separated from soldiers by a screen partition to prevent direct contact between the castes, workers continued to produce additional soldiers (Park and Raina, 2003). The regulation thus appears to be through feeding of soldiers by workers, i.e., trophallaxis. However, it is not clear what exact mechanism is involved in the suppression of their formation.

As stated earlier, the JH has been shown to induce soldier formation through the application of JH or JH analogs to workers. Although Haverty and Howard (1979) claimed that JHA such as methoprene and hydroprene did not induce pre-soldier differentiation in *C. formosanus*, its competence to molt to the pre-soldier is related to treatment dose, age of the worker, and seasonal variance. Our experiments with methoprene reaffirmed that exogenous JHA could induce soldier formation and considerably increase their population in a colony. While exercising a stimulatory effect on the workers, JHA treatment resulted in a significantly low JH III titer in young pre-soldiers (<3 day old). However, the JH titer of these pre-soldiers increased with age, even though it did not reach the

levels found in pre-soldiers produced normally without the application of an exogenous JHA. These results indicated that workers underwent a developmental transition influenced by external factors, and physiological changes followed immediately. In the cockroach *Diploptera punctata*, the depression of JH synthesis was noticed after application of exogenous JH (Tobe and Stay, 1979). *C. formosanus* workers at a low level of intrinsic JH titer could differentiate into pre-soldiers with the treatment of methoprene. However, the exogenous JHA seemed to be ineffective as a primary regulator to promote further differentiation of the pre-soldier. Our results indicated that intrinsic JH synthesis continued to occur in the JHA-induced pre-soldiers.

In general, *C. formosanus* colonies have about 10% soldiers. Some factors such as nest and environmental conditions result in the increase of soldier percentage in colonies. In the laboratory, the percentages of soldiers can reach as high as 25%, and up to 40% in response to JH or JHA treatment. However, based on Figs. 1 and 2, about 10% of workers became receptive to hormonal changes in response to a need to increase the soldiers. Higher than normal percentages of soldiers, then, trigger the feedback inhibition, thus preventing additional soldier formation. Further research is needed to fully understand the complexities of hormonal regulation of soldier formation in termites.

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